Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2013, 5(2):231-239

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Separation of Ditalimfos organophosphorus fungicide in adsorption thin layer chromatography and reverse phase high performance thin layer chromatography

P. M. Nagaraju[1,3], S. M. Bhargavi[1], U. S. Praveen[2] and K. Kemparaju[3*]

[1]*Regional Forensic Science Laboratory, Davangere, India*
[2]*Regional Forensic Science Laboratory, Gulberga, India*
[3]*Department of Studies in Biochemistry, Mysore University, Mysore, India*

ABSTRACT

Poisoning by ditalimfos (D) is although uncommon; recently, a few cases of deaths owing to the ingestion of ditalimfos were reported in our laboratory. The chromatographic separation is of primary interest when it is contemplated from a forensic matrix. Hence, a study was undertaken to separation of ditalimfos by NP-TLC on silica gel 60F_{254} and RP- HPTLC on silica gel 60RP-18 WF_{254} plates with varying mobile phase composition 10:0-6:4. The relationship between the mobile phase composition (n-hexane+acetone for NP-TLC, and methanol+water for RP-HPTLC) and R_F values affirmed that with the increase in the acetone content in case of NP-TLC and decrease of the water content in RP-HPTLC resulted in the increase in R_F values for the ditalimfos. The relationship between the mobile phase composition and retention parameter R_F, HR_F and R_M was analysed. Under the chromatographic condition ditalimfos has low RM values this indicate the hydrophilicity of the compound. The UV apex of maximum absorption was 200nm for ditalimfos as measured by multiwavelength scan in the UV range, and higher values of peak area and peak height was obtained at the same wavelength. The present study clearly indicate that separation of ditalimfos in different mobile phase composition in NP-TLC and RP-HPTLC.The minimum ΔR_F values indicate good separation was accepted as 0.04, when compared to the separation of any one neighbor compound. We can choose the any combination of solvent system where $\Delta R_F \geq 0.04$.

Key words: Ditalimfos, NP-TLC, RP-HPTLC, Mobile phase composition, Refraction factor

---

INTRODUCTION

Organophosphorus fungicides (OPF) were introduced in the mid to late 1960s to prevent or minimize crop losses caused by phytopathogenic fungi [1]. Although more than hundred organophosphorus compounds have fungicide action, relatively few (ampropylfos, ditalimfos, edifenfos, fosetyl-aluminum, hexylthiofos, iprobenfos, phosdiphen, pyrazophos, tolclofos-methyl and triamiphos) are of practical use. , ditalimfos (D), is commonly used by the farming community.

Ditalimfos (O, O-diethyl phthalimidophosphonothioate) is a non systemic foliar fungicide processing protects ant and curative biological activity [2]. It is used mainly to control powdery mildew on vegetables and fruit. Ditalimfos is relatively non toxic to mammals with an acute oral LD_{50} of about 4900 mg kg^{-1} bw [3]. It is having weak cholinergic activity, toxic to fish [4] as well as being considered as primary skin irritant [5]. Ditalimfos commonly used organophosporus fungicide; it is highly likely that is used for suicidal and homicidal purpose under extreme

conditions due to the crop losing. Therefore, it is a challenging task for forensic scientists to find out the exact fungicide used for the suicide and homicidal purpose.

It is imperative to study each new compound early in its development so that it may be permitted only for safe uses and under safe conditions. The preliminary study ought to identify the nature and degree of potential injury and to develop methods for screening such new compounds. Once a compound, whether old or new, is in use, it is essential to monitor its effects, especially on those most heavily exposed and newly introduced. Forensic toxicologists are dealing with maximum number of cases' due to organophosphate poisoning involved in various types of crimes. Since the analysis of residues poses an entirely different type of problem for the toxicologists because these residues are present in extremely small quantity in heterogeneous materials including the biological materials. The importance of residue problem led to intensive search for analytical methods for accurate and rapid analysis. The determination of the pesticide in various biological materials often faced with the problem of determining the minute quantities mixed with large amount of extraneous material or intermixing. Qualitative and quantitative methods are required to be applied keeping in view the sensitivity and specificity of the methods on one hand and nature and type of pesticides on the other hand [6, 7].

Several hundred organophosphates are available for use as insecticides, herbicides and fungicides and there is a need for systematic screening methods for use in forensic or clinical toxicology laboratories. The methods should be sensitive, specific, and applicable to the examination of proprietary formulations, food and drink, or biological fluids. Studies on the effect of ditalimfos on the biochemical parameters, availability of specific biomarkers for the diagnosis of poisoning and determination of residues and/or metabolites in biological fluids are limited. Hence, the study has been undertaken to evaluate the effect of routinely used ditalimfos on the biochemical parameters and to develop an affordable, sensitive and rapid chromatographic technique for the determination of residues and/or metabolites in forensic samples.

This study will put forensic toxicology in India on a new level and will greatly enhance the capability to screen forensic samples for ditalimfos poisoning cases. This multidisciplinary investigation combining forensic toxicology, clinical biochemistry and bioanalytical methods will create a unique platform needed by the forensic science laboratories and forensic medicine in unraveling the poisoning caused by ditalimfos.

Numerous authors have described the detection of parent compound of ditalimfos in various samples like crops, vegetables and fruit, wastewater and food. These include gas chromatography [8, 9], gas chromatography-mass spectrometry [10, 11], liquid chromatography-mass spectrometry [12, 13]. Most of these methods suffer from disadvantages of being time-consuming, requiring special procedures and sometimes derivatization.. Planar chromatography combined with automated instrumental techniques for sample application, plate development, and densitometric scanning of spots have allowed rapid and sensitive detection of various compounds free from minimum interference. HPTLC coupled with densitometry is simple, rapid, and of high analytical precision. It has advantages over other techniques because 10-20 samples can be separated in one run and small quantities of solvents are used. This reduces the time and cost of analysis. The separation of adsorption thin layer chromatography (NP-TLC) and reverse phase high performance thin layer chromatography (RP-HPTLC) is of primary interest when it is contemplated from a forensic matrix

EXPERIMENTAL SECTION

Instrumentation

HPTLC system: TLC scanner 3 (CAMAG, Muttenz, Switzerland), equipped with winCATS 1.4.2 software; sample application device: automatic TLC sampler 4 (ATS 4) (CAMAG); twin- trough glass chamber (CAMAG); and silica gel $60F_{254}$ HPTLC plates and Silica gel 60 RP-18 WF_{254} glass plates (Merck, Germany).

Reagents and chemicals

Ditalimfos (D) from Sigma–Aldrich was used as test solutes. The purities of the standards were >99%. Acetone (A), n-hexane (H), methanol (M) (Merck, Germany; analytical grade), and redistilled water (DW) were used as mobile phase components. Standard stock solutions (1ug mL^{-1}) of was prepared by dissolving an appropriate amount in methanol.

Preparation of Stock standard solutions
A standard stock solution (1 mg mL^{-1}) of ditalimfos was prepared by dissolving an appropriate amount in methanol. Working solutions of ditalimfos was prepared by diluting with methanol to get concentration of 0.1 µg mL^{-1}.

Chromatography
Adsorption Thin-Layer Chromatography (NP-TLC)
Adsorption Thin Layer Chromatography was performed on 10 cm × 10 cm aluminum plates precoated with silica gel $60F_{254}$. The plates were prewashed with methanol, dried for 24 h at room temperature and activated at 120°C for 30 min. Standard solutions were applied to the plates as 6 mm bands, 14 mm apart, 22 mm from the edges and 8 mm from bottoms of the plates, by use of a ATS 4 equipped with a 25-µL syringe and N_2 flow. The injection volume was 10 µL; sample-delivery speed was 100 nL s^{-1}. The plates were developed in a previously saturated glass twin-trough chamber, using *n*-hexane-acetone as mobile phase in volume compositions of 10:0, 9.5:0.5, 9:1, 8.5:1.5, 8:2, 7.5:2.5, 7:3, 6.5:3.5 and 6:4. The development distance was 8 cm from the lower edge of the plate. Photo documentation was by illumination of the plate at 254 nm and 366 nm and use of a CAMAG Reprostar 3 digital documentation system with built in high-resolution 12-bit CCD camera.

In-situ densitometric scanning was performed with a TLC Scanner 3, in absorbance mode at 254 nm using the deuterium light source. The slit dimension was 6.00 mm × 0.45 mm; scanning speed of 20 mm/s; data resolution 100 µm/step; and the optical filter was second order. Multi-wavelength scan was performed in the range of 200 to 300 nm at a wavelength increment of 10 nm at every step. Each track was scanned three times and baseline correction (lowest slope) was used.

Reverse Phase High Performance Thin Layer Chromatography (RP-HPTLC)
RP-HPTLC Chromatography was performed on 10 cm × 10 cm glass plates coated with Silica gel 60 RP-18 WF_{254} (Merck, Germany). The plates were prewashed with methanol, dried for 24 h at room temperature and activated at 120°C for 30 min. Standard solutions were applied to the plates as 6 mm bands, 14 mm apart, 22 mm from the edges and 8 mm from bottoms of the plates, by use of a Camag (Muttenz, Switzerland) ATS 4 equipped with a 25-µL syringe and N_2 flow. The injection volume was 10 µL; sample-delivery speed was 100 nL s^{-1}. The plates were developed in a previously saturated glass twin-trough chamber, using methanol-water as mobile phase in volume compositions of 10:0, 9.5:0.5, 9:1, 8.5:1.5, 8:2, 7.5:2.5, 7:3, 6.5:3.5 and 6:4. The development distance was 8 cm from the lower edge of the plate.

In-situ densitometric scanning was performed with a TLC Scanner 3 equipped with winCATS 1.4.2 software, in absorbance mode at 254 nm using the deuterium light source. The slit dimension was 6.00 mm × 0.45 mm; scanning speed of 20 mm/s; data resolution 100 µm/step; and the optical filter was second order. Multi-wavelength scan was performed in the range of 200 to 300 nm at a wavelength increment of 10 nm at every step. Each track was scanned three times and baseline correction (lowest slope) was used.

Chromatographic data
The chromatogram was done in triplicate and each track was scanned three times, and the mean of the R_F values were calculated. Next, the R_F values were recalculated on R_M values using this equation $K = \text{Log } 1\text{-}R_F / R_F$.

Retention parameters
Refraction factor (R_F)

$$R_F = \frac{\text{Distance moved by the solute from the origin}}{\text{Distance moved by the solvent front from the origin}}$$

R_F value vary from 1to 0, or from 100 to 0 if multiplied by 100 ($hR_F = R_F X100$)

Capacity Factor K, (R_M) is the ratio of the quantities of solute distributed between the mobile and stationary phases, or the ratio of the respective times the substance spends in the two phases. The capacity factor and R_F are calculated by the equation $K = \text{Log } 1\text{-}R_F / R_F$ [14-17].

RESULTS AND DISCUSSION

The organization of mobile phase selection parameters is based on the work of Snyder, which is summarized in his widely used monograph. The expression of resolution resulting from this and other work can prove very useful in choosing an appropriate mobile phase in both column and Thin Layer chromatography. Snyder's mathematical expressions of adsorption chromatography have made it possible to systematic choice of mobile phase. Solvent selectivity refers to the ability of a solvent to create differences in the relative migration of two solutes. For the separation of ditalimfos organophosphorus compounds on Adsorption Thin Layer Chromatography, mobile phase components consisted of n-hexane-acetone are widely used, and it is a first choice of solvent mixture to achieve separation on NP-TLC layers. In RP-HPTLC, the first-try solvent for C_8 or C_{18} layers is usually a solvent mixture composed of methanol-water. The other solvent mixtures used in RP-chromatography consisted of water plus an acetone, acetonitrile or dioxane. Hence, preliminary tests were performed with the objective of selecting optimum compositions of n-hexane-acetone and methanol-water as mobile phases for separation of ditalimfos NP-TLC and RP-HPTLC layers, respectively.

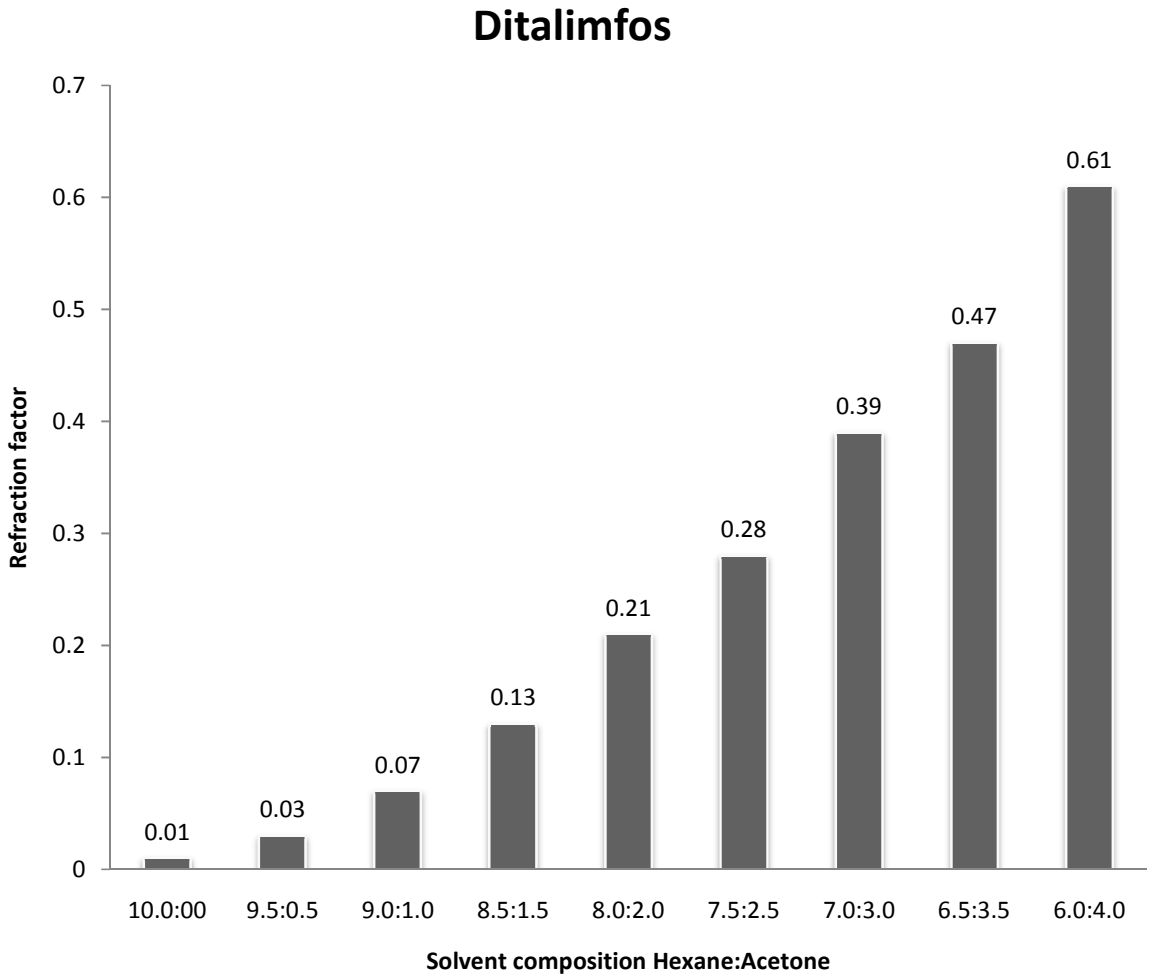

Fig.1: R_F values of ditalimfos in different volume fractions of n-hexane and acetone mobile phase at 254nm in NP-TLC

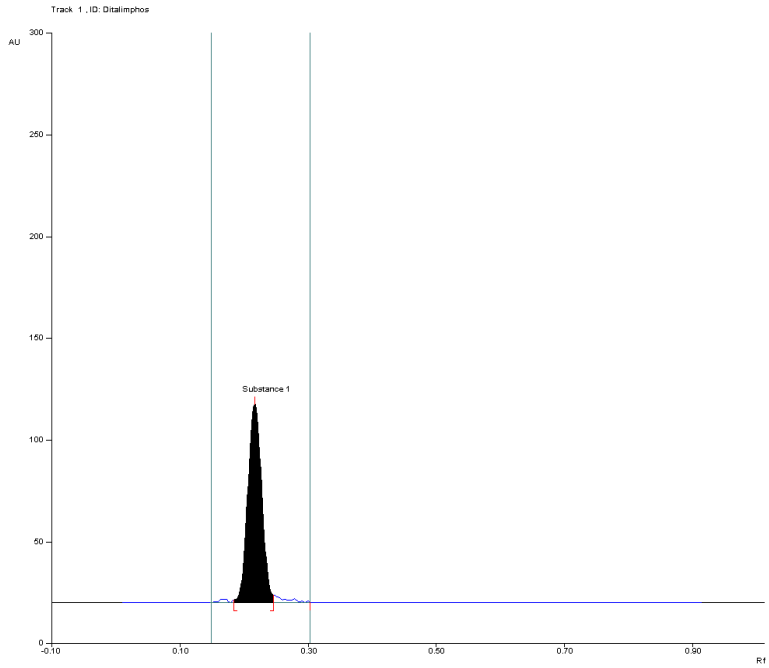

Fig.2: Densitogram of ditalimfos at 254 nm in the mobile phase n-hexane and acetone (8.0:2.0).

In adsorption Thin Layer Chromatography preliminary test was performed with the objective of selecting the optimum volume composition of n-hexane-acetone as mobile phase for the separation of ditalimfos on silica $60F_{254}$. Results obtained using n-hexane-acetone as mobile phase was analyzed as relationship between R_F values and mobile phase composition. It was found that the R_F values of the ditalimfos increase with an increase in acetone content in the mobile phase. With the increase of acetone content in the mobile phase, gradual increase in the R_F values can be observed for D. This was depicted in as plot of R_F against volume composition of n-hexane-acetone mobile phase for the D at 254nm is shown in fig.1. Increase the polar solvent in the mobile phase increase the R_F and decrease the retention time. The densitogram obtained at 254 nm for the ditalimfos by use of mobile phase composition n-hexane:acetone in the ratio 8.0:2.0 is shown in fig .2.

In Reverse Phase High Performance Thin Layer Chromatography (RP-HPTLC) for the separation of ditalimfos on RP-18 WF_{254} plates. Hence, methanol-water in different volume compositions was studied for the effective separation of the studied D. Results obtained using methanol-water as mobile phase was studied as relationship between R_F values and mobile phase composition. It was found that with the increase of water content in the mobile phase, gradual decrease in the R_F values can be observed for D (Fig.3). The densitogram of the ditalimfos at 254 nm using methanol: water mobile phase with a volume composition of 8:2 is presented in Fig. 4. The mobile phase volume composition 10:0 to 6:4 in NP-TLC and RP-HPTLC have different peak area and peak heights, the data presented in Table 1 and 2 respectively.

Table No.1- Retention parameters obtained for Ditalimfos in different volume composition of n-hexane: acetone at 254nm in Adsorption Thin Layer Chromatography

Solvent System Hexane:Acetone	R_F in triplicate	hR_F	Capacity factor (R_M)	Peak height	Peak area
10:00	0.01	1	1.995	102.9	704.3
9.5:0.5	0.03	3	1.510	147.5	981.5
9.0:1.0	0.07	7	1.123	113.5	1859.0
8.5:1.5	0.13	13	0.825	112.5	2505.5
8.0:2.0	0.21	21	0.575	102.6	4251.1
7.5:2.5	0.28	28	0.401	105.1	2329.8
7.0:3.0	0.39	39	0.194	123.7	3053.5
6.5:3.5	0.47	47	0.052	103.5	2926.7
6.0:4.0	0.61	61	-0.194	99.7	2947.9

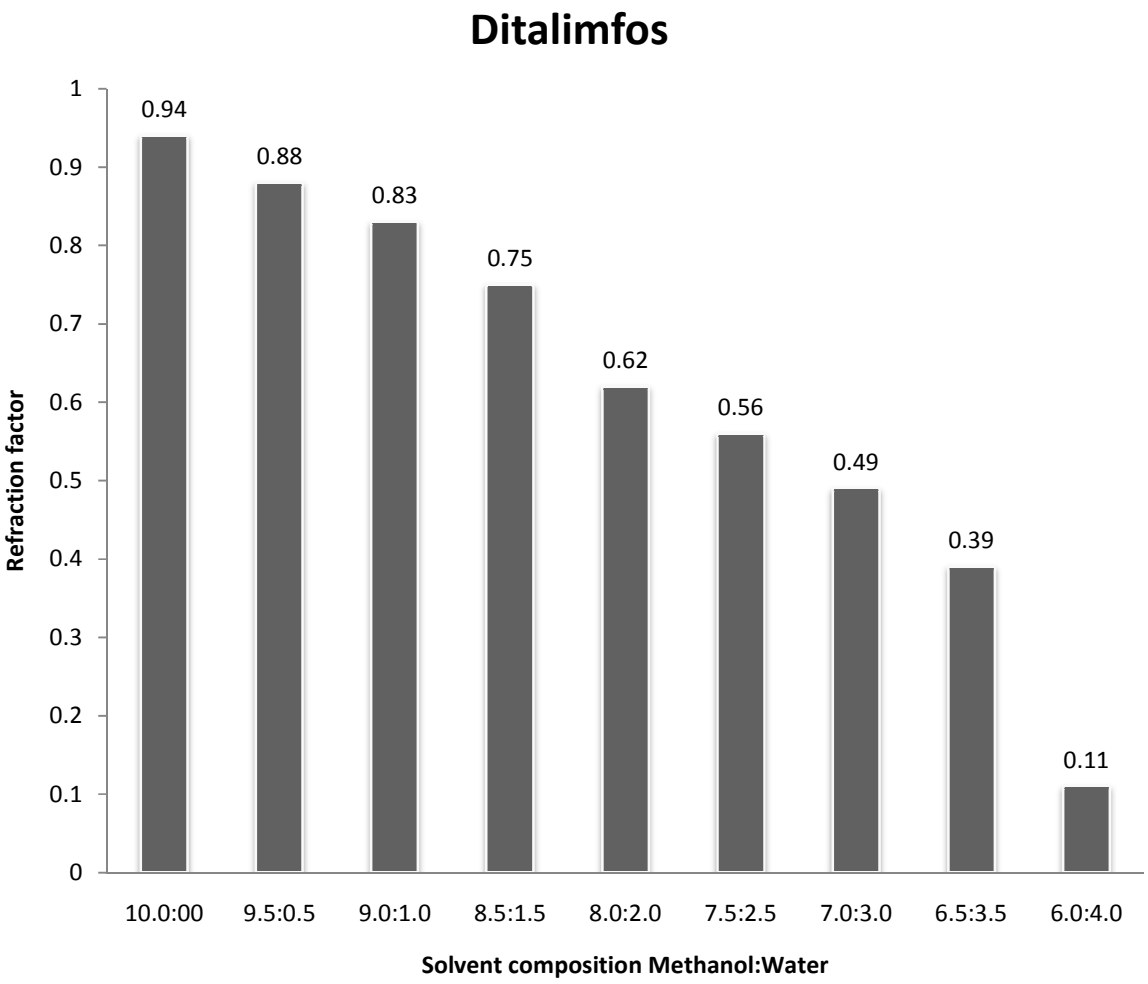

Fig.3: R_F values of ditalimfos in different volume fractions of methanol and distilled water mobile phase at 254nm in RP-HPTLC

Table No.2- Retention parameters obtained for ditalimfos in different volume composition of methanol: water at 254nm in RP-HPTLC.

Solvent System Methanol:water	R_F in triplicate	hR_F	Capacity factor (R_M)	Peak height	Peak area
10:00	0.94	94	-1.195	61.6	1594.4
9.5:0.5	0.88	88	-0.865	98.1	2773.4
9.0:1.0	0.83	83	-0.690	75.8	2289.0
8.5:1.5	0.75	75	-0.477	66.1	2052.2
8.0:2.0	0.62	62	-0.213	75.5	2513.9
7.5:2.5	0.56	56	-0.105	55.1	2657.8
7.0:3.0	0.49	49	0.017	55.5	2793.9
6.5:3.5	0.39	39	0.194	82.7	2984.0
6.0:4.0	0.11	11	0.907	79.3	1263.8

In a further part of this work, UV apex of maximum absorption for the ditalimfos was confirmed by acquiring multiwavelength scans in the range 200 to 300 nm, with a wavelength increment of 10 nm per step. The plates developed in n-hexane-acetone 8:2 (v/v) and methanol-water 8:2 (v/v), were used for this purpose. The heights and areas of chromatographic band were recorded for ditalimfos in the wavelength range 200 to 300 nm. The results obtained are presented in Table 3. The heights and areas of the chromatographic bands obtained for the ditalimfos at different wavelengths have different values. However, maximum height and area were obtained at 200 nm. The multiwave length densitogram of ditalimfos in NP-TLC and RP-HPTLC presented in figure 5 and 6 respectively. In-

situ UV absorption spectrum was recorded for ditalimfos and the UV apex of maximum absorption was found to be 200 nm.

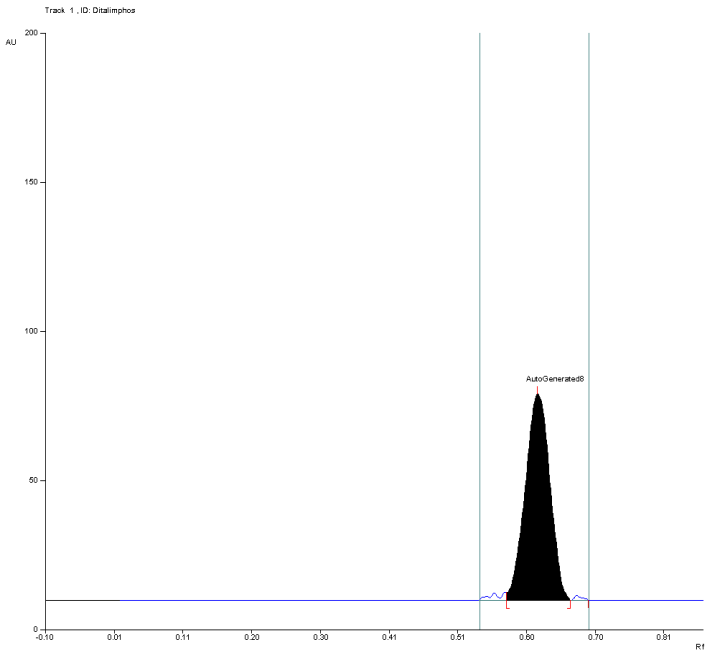

Fig.4: Densitogram of ditalimfos at 254 nm in the mobile phase methanol and distilled water (8.0:2.0).

Table No. 3- Heights and areas of densitometric bands obtained for ditalimfos, in the wavelength range 200 to 300 nm in NP-TLC and RP-HPTLC

wavelength	n-hexane: acetone 8:2 (v/v) NP-TLC		Methanol:water 8:2 (v/v) RP-HPTLC	
	Height (AU)	Area (AU)	Height (AU)	Area (AU)
λ 200nm	329.2	11587.4	446.1	8157.3
λ 210nm	264.0	9002.7	403.0	7043.4
λ 220nm	231.5	7677.3	382.0	6515.6
λ 230nm	212.2	6995.2	371.1	6334.0
λ 240nm	180.2	5874.6	321.2	5408.2
λ 250nm	123.6	3941.9	221.2	3608.6
λ 260nm	78.8	2455.1	180.6	2909.3
λ 270nm	73.6	2290.2	200.6	3276.5
λ 280nm	70.6	2218.8	199.1	3295.9
λ 290nm	47.8	1504.2	122.3	2025.9
λ 300nm	19.2	621.6	40.5	666.1

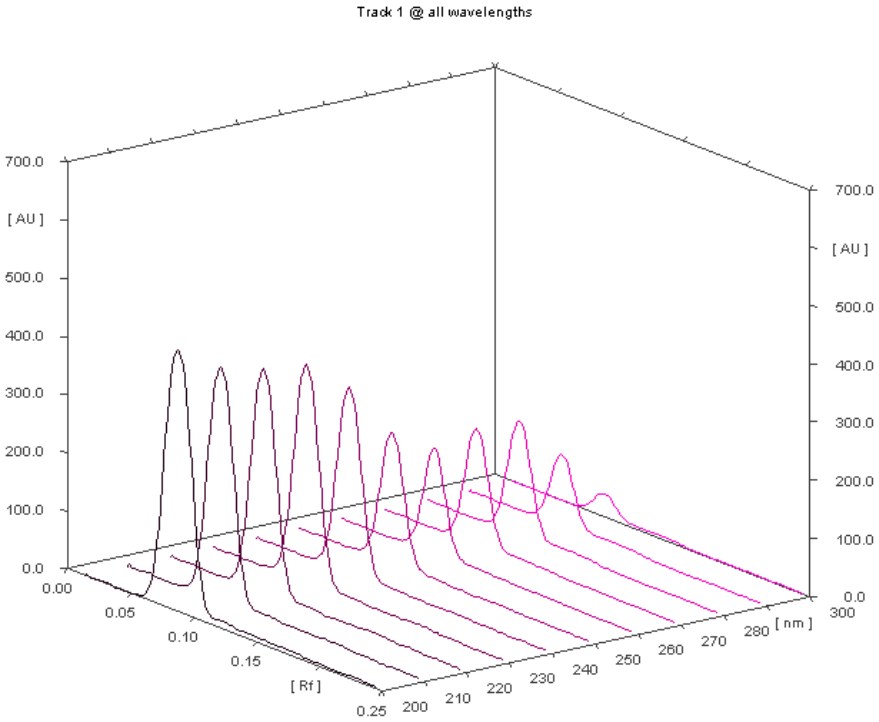

Fig.5: Multiwave length densitometric bands obtained for ditalimfos, in the wavelength range 200 to 300 nm in Adsorption Thin Layer Chromatography

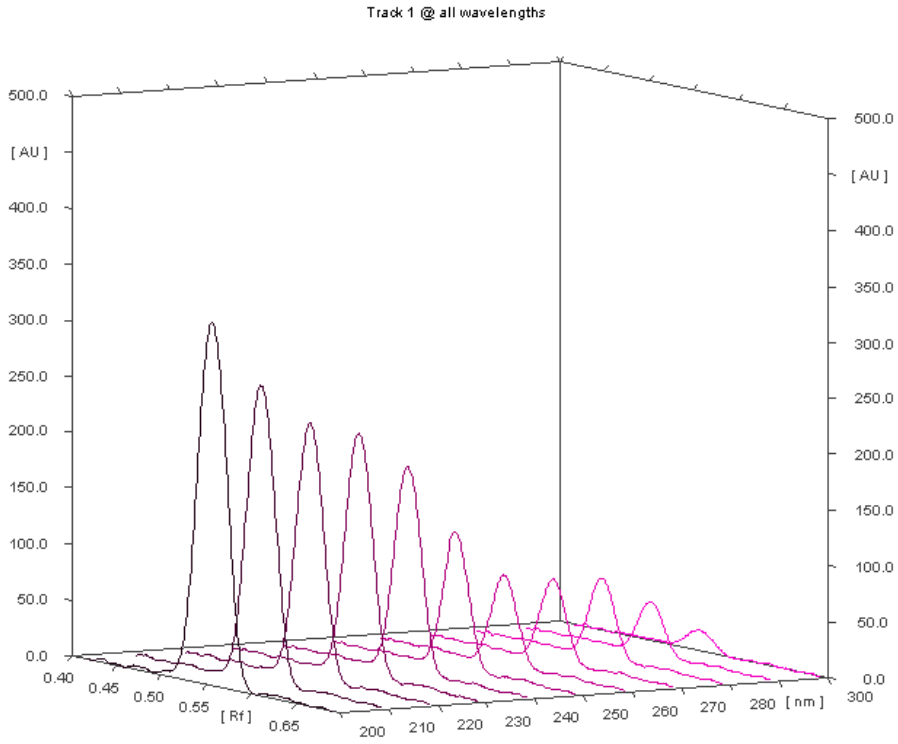

Fig.6: Multiwave length densitometric bands obtained for ditalimfos, in the wavelength range 200 to 300 nm in RP-HPTLC

CONCLUSION

A simple HPTLC technique is used for the separation ditalimfos on silica gel F_{254} and RP-18 WF_{254} plates. NP-TLC is widely used for the separation of ditalimfos than RP- HPTLC techniques. In RP-HPTLC is rarely used, it can determine the hydrophobicity of the particular compound. In NP-TLC the mobile volume composition n-hexane: acetone in the range 10.0:0.0 to 6.0:4.0 was used. Where increasing solvent strength decreases retention and increases the R_F value. Depending on the retention mechanism, solvent strength can have various reasons and descriptors. In adsorption thin layer chromatography, solvent strength is generally regarded as "polarity". The minimum ΔR_F values indicate good separation was accepted as 0.04, when compared to the separation of any one neighbor compound. We can choose the any combination of solvent system where $\Delta R_F \geq 0.04$[18]. $\Delta R_F = R_{F1}-R_{F2}$ where $R_{F1} > R_{F2}$, R_{F1} and R_{F2} are the R_F values of two adjacent peaks on the densitogram.The optimized technique, not only finds it use for the separation of ditalimfos, but also for the specific detection by use of obtained in-situ UV absorption spectra. The use of visualizing reagents for the detection of ditalimfos can be eliminated, as most of the reagents are not specific for a particular compound.

Acknowledgement
Authors are thankful to B. N. Kadaramandalagi, Deputy Director, Regional Forensic Science Laboratory, Davanagere.

REFERENCES

[1] de Waard, M.A. Mechanisms of action of the organophosphorus fungicide pyrazophos. *WAU dissertation* **1974,** 596.
[2] H Tolkmith. *Nature*. **1996,**.211, 522-523.
[3] http://sitem.herts.ac.uk/aeru/iupac/Reports (Ref: DOWCO 199)
[4] D Hartley, H. Kidd, Agrochemicals handbook. Royal chemical society, Nottingham, England, **1983**, pp. A168.
[5] KS Rao, JE Besto, KJ Olson, *Drug Chem Toxicol*. **1981**, **4** 331-351.
[6] K Kaur, RK Garg; Anil Aggrawal's *Internet Journal of Forensic Medicine and Toxicology*. **2003**, 4.
[7] PM Nagaraju, PU Sanganalmath; K Kemparaju; BM Mohan. *Journal of Planar Chromatography*. **2011,** 24 (2), 108-112.
[8] H Obana, K Kikuchi; M Okihashi; S Hori. *Analyst*. **1997,** 122, 217-220.
[9] K Sasaki, T Suzuki; Y Saito. *J. AOAC*. **1987,** 70 (3), 460-464.
[10] O Yukiko, Y Takashi; N Takeshi; T Toshiaki. *Analytical Sciences*. **2006,** 22, 1473-1476.
[11] F Musshoff, H Junker; B Madea. *J. Chromatogr Sci***.2002**, 40 (1), 29-34.
[12] C Blasco, G Font; Y Pico. *Anal Chem*. .**2003**, 75 (14), 3606-3615
[13] C Blasco, Y Pico; J Manes; G Font. *J. Chromatogr Sci*.**2002**. 947 (2): 227-235.
[14] G Bazylak, H Brozik; W Sabanty. *Polish Journal of Environmental Studies*.**2000**, 9, 113-123.
[15] C Sarbu, M A Demertzis; D Kovala. *J. Acta Chromatogr*.**2000**, 10, 222-229 .
[16] M M Acanski, SJ Santa; and LR Jevric. *J. Serb.Chem.Soc.*, **2003**, 68, 57-63.
[17] J Sherma, B Fried, Handbook of Thin-layer Chromatography, Edition 3, CRC press, **2003**.
[18] M Miszczyk, A. Pyka. *J. Planar Chromatogr*.**2006** 19, 15-20 (2006).